HAYATI Journal of Biosciences 24 (2017) 124–130

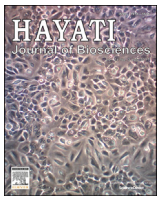
HOSTED BY

ELSEVIER

Contents lists available at ScienceDirect

HAYATI Journal of Biosciences

journal homepage: http://www.journals.elsevier.com/hayati-journal-of-biosciences



Original Research Article

Food Origin Fibrinolytic Enzyme With Multiple Actions

Laurentia Stephani,[1,2] Raymond Rubianto Tjandrawinata,[3] Diana Nur Afifah,[1] Yanti Lim,[2] Wangsa Tirta Ismaya,[3] Maggy Thenawidjaja Suhartono[1*]

[1] *Department of Food Science and Technology, Bogor Agricultural University, Darmaga Campus Bogor, Indonesia.*
[2] *Faculty of Biotechnology, Atmajaya University, Jakarta, Indonesia.*
[3] *Dexa Laboratories of Biomolecular Sciences, Dexa Medica Jababeka Cikarang, Jakarta, Indonesia.*

ARTICLE INFO

Article history:
Received 19 May 2017
Received in revised form
29 August 2017
Accepted 3 September 2017
Available online 29 September 2017

KEYWORDS:
enzyme,
fibrinolytic,
Oncom,
Stenotrophomonas

ABSTRACT

Many health related problems such as cardiovascular diseases are associated with the formation of excessive clot in the blood (thrombus). Approaches in cardiovascular disease treatment are preventing the formation or removing the thrombus. The present thrombolytic agents can be classified as plasminogen activators, fibrinolytic enzyme which directly degrades fibrinogen or fibrin and heparin type which act as thrombin inhibitor. Recently, microbial fibrinolytic enzymes of food origin receive more attention that leads to escalating efforts to explore traditional fermented foods as the natural sources. We have successfully isolated microorganism from Indonesian fermented soybean tofu dregs "*Oncom*" that secretes fibrinolytic enzyme. The microorganism identified as *Stenotrophomonas* sp. is unique because most of the reported fibrinolytic microorganism belongs to *Bacillus* sp. This isolate was found to produce extracellular fibrinolytic enzyme which could degrade fibrinogen and fibrin directly as determined by fibrinogen zymography and fibrin plate methods. More importantly, the 30-kD purified enzymes was found to demonstrate not only fibrin and fibrinogen degradation capabilities, but also acted as thrombin inhibitor as determined using specific substrates for thrombin. This is the first report of a fibrinolytic enzyme that demonstrates additional synergistic activities. This finding accentuates the importance of further development of the enzyme into a powerful agent to treat the thrombus-related disease effectively.

1. Introduction

A variety of diseases and health problems are influenced by reactions that maintain a balance between blood coagulation and anticoagulation. Coagulation process produces fibrin-containing blood clots which is formed from fibrinogen by thrombin catalysis (Lioudaki and Ganotakis 2010; Voet and Voet 1990; Wang *et al.* 2006). Fibrin can be dissolved by fibrinolytic enzymes such as plasmin, which is normally activated from the nonactive plasminogen by a tissue-type plasminogen activator (tPA) (Collen and Lijnen 2004; Nakajima *et al.* 1993; Wang *et al.* 2011). This process maintains blood flow at vascular injury sites and is an important component of the normal haemostatic responses. Disturbances in the anticoagulation process can lead to accumulation of fibrin in the blood vessels and results as thrombosis conditions which usually lead to myocardial infarction and other cardiovascular diseases (Collen and Lijnen 2004; Kim *et al.* 1996). Researches are continuously pursued to find fibrinolytic agents, plasminogen activators and thrombin inhibitors which are safe and can work efficiently.

Tissue plasminogen activator (tPA) is a serine protease which catalyses the conversion of plasminogen to plasmin, a major enzyme responsible for breakdown of fibrin in the blood clots. Plasminogen activators, such as tPA, urokinase, alteplase and reteplase are used in the clinical medicine to treat embolic and thrombotic strokes (Dubey *et al.* 2011; Duffy 2002; deMers 2012). Thrombin has many important functions in the clotting pathway leading to formation of the insoluble fibrin clots, so it is a good target for anticoagulants drugs. Natural antithrombin (AT) is a small protein molecule of 58 kDa that inactivates several enzymes of the coagulation system. ATs are required not only for blood disease disorder, arterial and deep vein thrombosis or coronary syndromes, but also during sepsis conditions which is known to activate the

* Corresponding author.
E-mail address: mthenawidjaja@yahoo.com (M.T. Suhartono).
Peer review under responsibility of Institut Pertanian Bogor.

https://doi.org/10.1016/j.hjb.2017.09.003

coagulation system and induces intravascular fibrin coagulation associated with increased mortality. Some of the clinically approved antithrombotic drugs include natural protein AT purified from human blood, recombinant human AT, hirudin peptides anticoagulant heparin and its derivative. Their mode of actions range from hydrolysing AT or bind at specific sites of the thrombin molecules, resulting in the inactivation of the thrombin action (Fareed *et al.* 1999; Li *et al.* 2004).

Microbial fibrinolytic enzymes from food origin attracted much more medical interest lately. Many indigenous fermented foods have the ability to prevent thrombosis. A number of potent fibrinolytic enzymes have been isolated and characterized from fermented food products, such as Korean *Chungkook-Jang* (Kim *et al.* 1996), Japanese Natto (Wang *et al.* 2009), Chinese Douchi (Wang *et al.* 2006) and Indonesian Tempe (fermented soybean; Kim *et al.* 2006; Sugimoto *et al.* 2007). The fibrinolytic enzymes obtained from different microorganisms were mostly of the genus *Bacillus*.

We have successfully isolated a microorganism from Indonesian fermented soybean/tofu dregs "*Oncom*" that secretes fibrinolytic enzyme. The microorganism was identified as *Stenotrophomonas* sp., which is unique because most of the reported fibrinolytic microorganism belongs to *Bacillus* sp. We had confirmed safety of the extracellular protein enzyme of the isolate 11 (*Stenotrophomonas* sp.) using cell culture and experimental rat; in addition, the thrombus degrading effect of the enzyme had been also tested using experimental rats (Nailufar *et al.* 2016). This study showed that the purified extracellular fibrinolytic enzyme has multiple activities, namely degrading fibrinogen and fibrin directly as well as acting as AT. This is the first report on a fibrinolytic enzyme that demonstrates additional synergistic activities. This finding accentuates the importance of further development of the enzyme into a powerful agent to treat the thrombus-related disease effectively.

2. Materials and Methods

2.1. Microorganisms

Bacterial isolates used as source of fibrinolytic enzymes were obtained and screened from the red *oncom*, a fermented soybean/tofu dregs. Identification of the specific isolate was based on 16s rDNA gene sequence analysis.

2.2. Fibrinolytic enzyme production

The medium used for cultivation of the bacterial isolate and enzyme production was described by Miyaji *et al.* (2005), which consisted of 0.5% (w/v) casein, 0.5% (w/v) glucose, 0.6% (w/v) $Na_2HPO_4.2H_2O$, 0.2% (w/v) yeast extract, 0.1% (w/v) KCl and 0.01% (w/v) $MgSO_4.7H_2O$. pH of the medium was adjusted to 8.1 with 0.1 M sodium carbonate buffer. The cultivation was performed aerobically in a shaker incubator at 120 rpm for 48 h. The crude enzyme was obtained as supernatant following centrifugation at 9000*g*, 4°C for 30 min.

2.3. Fibrin plate assay

The fibrinolytic activity was measured using the standard fibrin plate method (Astrup and Mullertz 1952) with modification. In a petri dish (diameter of 85 mm), 7.3 mL of 0.5% w/v agarose and 200 μL of 1 mg/mL bovine thrombin were mixed gently, 2.5 mL of 1% w/v bovine fibrinogen was added and mixed to induce a solid fibrin formation. The fibrin plate was incubated at 37°C after 10 μL of the enzyme sample was dropped onto the disc (6 mm) previously placed on the surface of the fibrin agar. Diameter of the clearing zone areas were measured, and indicated as fibrinolytic activity of the enzyme.

2.4. Fibrin degrading unit

Fibrinolytic activity was measured using fibrin degradation assay described earlier (Hua *et al.* 2008). As much as 0.4 mL of 0.72% fibrinogen was placed in a test tube with 0.1 mL of 245 mM phosphate buffer (pH 7) and incubated at 37°C for 5 min. Then, 0.1 mL of a 20 U/mL thrombin solution was added. The solution was incubated at 37°C for 10 min, 0.1 mL of diluted enzyme solution was added, and incubation was continued at 37°C. This solution was shaked again after 20 and 40 min incubation. At 60 min, as much as 0.7 mL of 0.2 M trichloroacetic acid was added and mixed. The reaction mixture was centrifuged at 15,000*g* for 10 min. Then, 1 mL of the supernatant was collected and the absorbance at 275 nm was measured. In this assay, 1 unit (fibrin degradation unit, FU) of enzyme activity is defined as a 0.01 increase in absorbance at 275 nm/min.

2.5. Protein determination

Protein concentration was determined by Lowry's method using bovine serum albumin as a protein standard (Lowry *et al.* 1951).

2.6. Ammonium sulphate precipitation

Ammonium sulphate was added to the crude enzyme solution (free extracellular supernatant) at 4°C with continuous stirring over night to get saturation percentage of 65%, then centrifuged at 16,000 *g* at 4°C for 15 min. The precipitate was collected and dissolved in a half amount of 0.2 M phosphate buffer pH 7.5 from the initial volume. The dissolved enzyme was desalted against the same buffer using HiTrap Desalting column (GE Healthcare Chicago USA).

2.7. Enzyme purification

Protein solution from the desalting process was loaded onto HiTrap DEAE Sepharose column (GE Healthcare Chicago USA) equilibrated with 20 mM phosphate buffer, pH 7.5. The column was washed with the same buffer and the proteins were eluted using the same buffer containing NaCl (0—1.0) M by a stepwise gradient of 0.15 M, 0.35 M, 0.50 M and 1.0 M NaCl at a flow rate of 1 mL/min.

2.8. Thrombin inhibition assay

Chromozym TH (Roche Applied Science Penzberg Germany) used for thrombin activity assay was dissolved in redistilled water. The reaction was performed by adding 2.8 mL of Tris buffer (0.05 M, pH 8.3), 227 mM NaCl and 0.3 mL Chromozym TH (1.9 mM) into the cuvette. The enzyme sample was added at 0.1 mL, and the absorbance at 405 nm was observed. Changes in absorbance: ΔA/min was calculated from the linear curve. Under these conditions, the enzyme activity (U/mL) can be calculated as 3.077 × ΔA/min by following the instruction manual.

2.9. SDS-PAGE (Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis) and zymogram

SDS-PAGE was performed using Laemmli's method (Laemmli 1970) with 12% separating gel and 4% stacking gel. The gel was run at 70 V, 400 mA for 2 h. The protein bands were then visualized by staining the gel with Coomassie blue and the apparent molecular mass of the protein was calculated using low molecular weight standard protein markers (GE Healthcare).

3. Results

Oncom is a traditional fermented soybean/tofu dregs. The soy protein soft material used for the basic ingredient comes from the bean paste left during tofu making, and this made the typical soft texture in the red *oncom*. The red colour is due to the metabolism product of the fungi, indicating high content of beta carotene.

Indonesian people recognized *Oncom* as a traditional food rich in vitamin B and has ability to reduce cholesterol. We used *Oncom* to screen for fibrinolytic bacteria and found one of the potent isolates identified as *Stenotrophomonas* sp. The extracellular enzyme was capable of degrading fibrinogen and fibrin substrates. In our previous study, we had confirmed safety of the extracellular protein enzyme produced by this bacterium using cell culture and experimental rat. In addition, the thrombus degrading effect of the enzyme had been also tested using experimental rats (Nailufar *et al.* 2016)

3.1. Comparison of fibrinolytic activity

Isolate *Stenotrophomonas* sp. was grown in the media as mentioned in the method. The extracellular fibrinolytic enzyme (ST) was precipitated by 65% ammonium sulphate and dialysed against phosphate buffer 20 mM, pH 7.5 using HiTrap Desalting column. The fibrinolytic activity of the bacteria was compared with the commercial fibrinolytic enzyme, lumbrokinase (LK), a plasminogen activator originated from earthworm, which has been used as medical agents for thrombosis. Both were used at concentration 40 mg/mL for the fibrin plate analysis. Fibrinolytic activity was shown as clearing zone in the fibrin plate. The result indicated that fibrinolytic activity from *Stenotrophomonas* sp. was comparable with that of the commercial LK (Figure 1).

3.2. Purification of fibrinolytic enzyme

Enzyme in the cell-free supernatant (crude) was precipitated by addition of 65% ammonium sulphate. The pellet was collected, desalted with HiTrap Desalting column, and washed with 20 mM phosphate buffer, pH 7.5. The enzyme was then passed through a DEAE Sepharose column, and washed with 20 mM phosphate buffer, pH 7.5. The attached proteins were eluted with a stepwise gradient of 0.15 M, 0.35 M, 0.50 M and 1.0 M of NaCl in the same buffer at a flow rate of 1 mL/min. Some unbound proteins eluted is shown as peak I (Figure 2). Peaks II and III were eluted in the presence of higher NaCl concentrations. All fractions were analysed using fibrin degrading unit assay and peak III was found to have fibrinolytic activity. The enzyme was purified 8.2-fold.

3.3. SDS-PAGE analysis

The crude enzyme appeared as five protein bands in the SDS PAGE electrogram, with apparent molecular weight of 19–64 kDa. Peak II was found as 64-kDa protein, peak IIIa as 30-kDa protein, peak IIIb as 19-kDa protein, whereas peak IV did not show observable protein band and peak I produced smeared protein below 20 kDa (Figure 3).

3.4. Antithrombin activity

Chromozym TH (Tos-Gly-Pro-Arg-4-nitranilide acetate) was used as a specific substrate for assay the thrombin activity. The capability of thrombin to hydrolyse this substrate was shown as increase in absorbance at 405 nm (Figure 4). When the crude enzyme was mixed with thrombin (Figure 4A), the thrombin activities were not altered at the time applied. The result was different when we incubated thrombin with the purified enzyme (peak IIIa). Thrombin inhibition was markedly increased after 24 h incubation (Figure 4B). We observed much more significant decrease in the absorbance at 405 nm compared with the absorbance shown by thrombin alone. The purified enzyme (peak IIIa) itself did not capable of hydrolysing the thrombin substrate. This implies that the purified enzyme acted as an inhibitor of thrombin or behaved as an AT.

4. Discussion

Enzyme therapies are becoming more prevalent in the medical world today, with many manufacturers targeting their advantages in disease treatment. In the past, the therapeutic use of enzymes has been largely focused on the use of digestive enzyme. Recently, however, many new enzymes are reported to have metabolic effects and to work directly in concert with the natural metabolic enzymes. Natural enzymes with fibrinolytic nature has been found potential, that even could rival pharmaceutical agents in thrombosis treatment and show promises in related areas such as cardiovascular diseases, stroke, angina, thrombosis, emboli, atherosclerosis, rheumatism, chronic inflammation, hypertension etc.

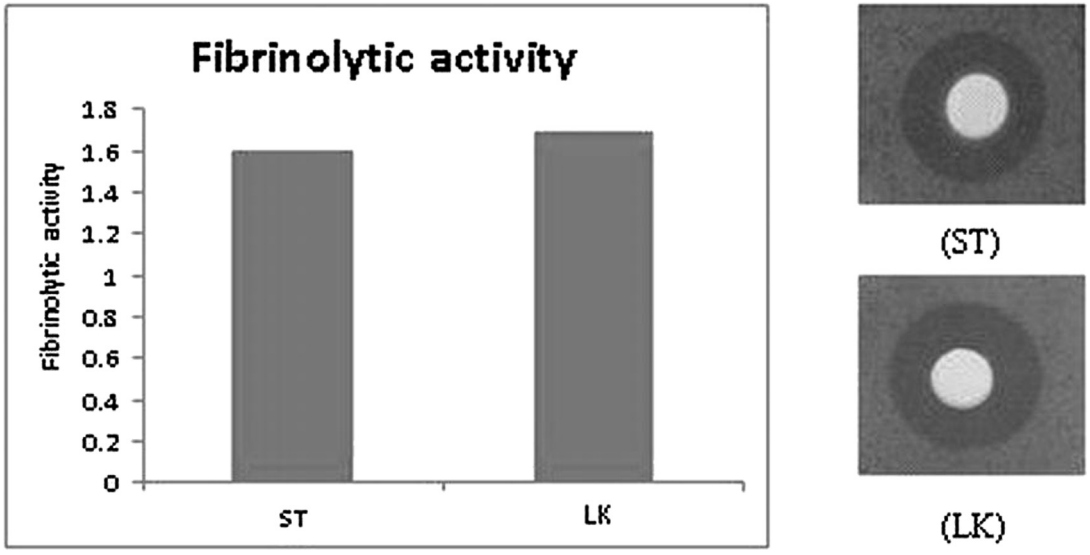


Figure 1. Comparison of fibrin-degrading enzyme of extracellular protease from *Stenotrophomonas* sp. (ST) and the commercial product of lumbrokinase (LK). Comparison of fibrin degrading enzyme activity was based on the fibrin plate method as described in Figure 2. Diameter of the hydrolysed clear zone was measured and calculated as fibrinolytic activity of the samples. The samples were applied at 40 mg/mL. Extracellular crude enzyme from *Stenotrophomonas* sp. (ST). Commercial fibrinolytic agent from *Lumbricus rubellus* (LK).

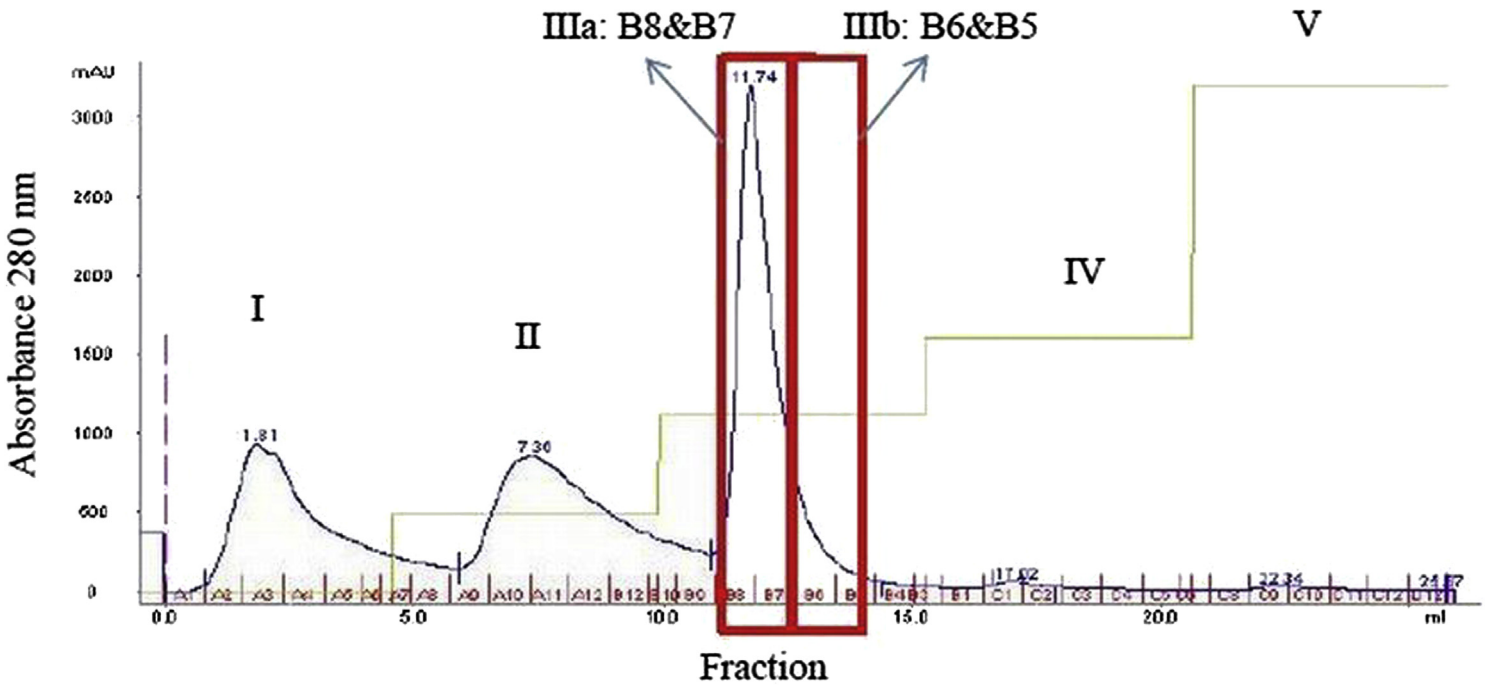


Figure 2. Detection of proteins eluted from HiTrap DEAE FF column. Purification of fibrinolytic enzyme was performed by anion exchange chromatography (HiTrap DEAE FF column). After precipitation with ammonium sulphate 65% and dialysis, the protein sample was loaded onto the column, washed with 20 mM phosphate buffer, pH 7.5. The attached proteins were eluted with a stepwise gradient of 0.15 M, 0.35 M, 0.50 M and 1.0 M of NaCl in the same buffer at a flow rate of 1 mL/min. Unbound eluted protein (peak I). Attached protein eluted by buffer containing 0.15 M NaCl (peak II), 0.35 M (peak IIIa), 0.35 M (peak IIIb), 0.50 M (peak IV) and 1.0 M (peak V).

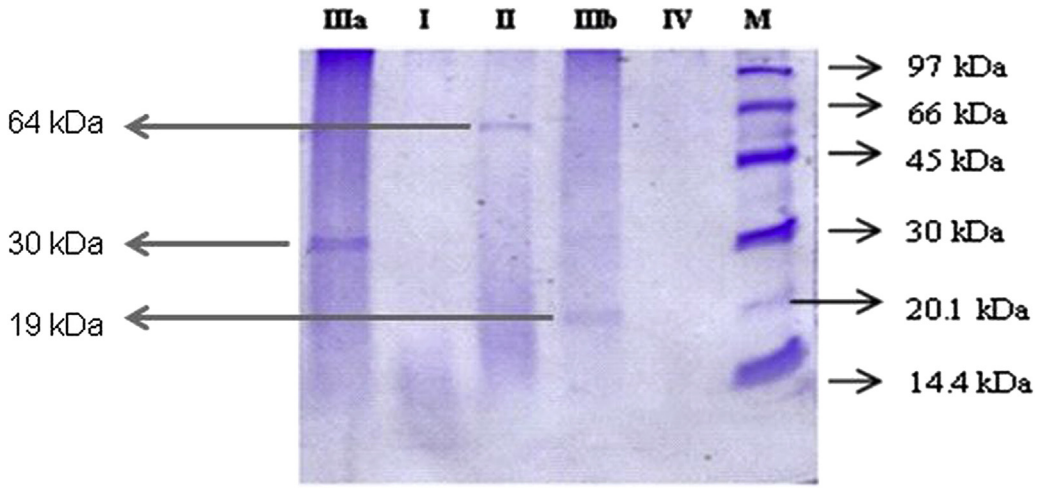


Figure 3. SDS-PAGE analysis. SDS-PAGE was conducted according to the Laemmli's method (1970). Samples were loaded onto the stacking gel (4%), proteins were resolved in 12% polyacrylamide gel (resolving gel). Electrophoresis was conducted at 70 V and stained with Coomassie brilliant blue solution. Low molecular weight standard protein markers (GE Healthcare) were used to predict the molecular weight of the proteins. Bands of unbound protein (peak I) and purified protein (peaks II, IIIa, IIIb and IV) are shown.

In this study, the primary target of the enzymatic therapy is the protein fibrin, which is a final product in blood clotting cascade processes; it is derived from its soluble protein precursor, fibrinogen. Fibrinolytic (thrombolytic) agents for therapy are used to activate the fibrinolytic system by converting the inactive proenzyme, plasminogen into the active enzyme plasmin, that degrades the fibrin target. The following are the agents available presently for the clinical use: the physiologic tPA, urokinase-type plasminogen activator and the bacterial plasminogen activator such as streptokinase. Despite their widespread use, these agents suffer from several significant limitations, including bleeding complications. Several lines of research towards improvement of thrombolytic agents are being explored, including search for plasminogen activators of safer origin. The finding of a potent fibrinolytic enzyme Nattokinase from *Bacillus natto* in the Japanese traditional fermented food Natto known for decades by the Japanese as nutritious and healthy food has opened up possibilities of safer oral treatment to overcome thrombosis This finding has motivated active searching of numerous fibrinolytic microorganisms in the traditional (Asian) fermented food. These studies revealed that, indeed, food, especially the fermented food is considered as one of the abundant sources of fibrinolytic enzymes. Studies of microbes producing fibrinolytic enzymes from various traditional Asian fermented foods have also reported that these microorganisms were mostly of the genus *Bacillus* (Kim and Choi 2000; Kim *et al.* 1997; Peng *et al.* 2003).

In our study, bacteria isolated from red *Oncom*, a soybean/tofu dregs fermented product were screened for its fibrinogenolytic and fibrinolytic activities and three potential isolates were identified as *Bacillus licheniformis*, *Bacillus cereus* and *Stenotrophomonas* sp. *B. licheniformis* and *B. cereus* only showed either fibrinogenolytic or fibrinolytic activity, whereas *Stenotrophomonas* sp. interestingly showed both fibrinogenolytic and fibrinolytic activity. An agent which can degrade both fibrinogen and fibrin will be more effective for thrombosis treatment. We had confirmed safety of the extracellular protein enzyme of the isolate *Stenotrophomonas* sp. using cell culture and experimental rat; in addition, the thrombus degrading effect of the enzyme had been also tested using experimental rats (Nailufar *et al.* 2016). This result encouraged us to further purify and analyse the enzyme from this isolate. Finding of food origin fibrinolytic *Stenotrophomonas* sp. is considered unique and surprising because most of the reported fibrinolytic microorganism from fermented food belongs to *Bacillus* sp.

Genus *Stenotrophomonas* is not yet well explored, but has been associated with variety of functions and applications that are beneficial such as plant growth, bio- and phytoremediation and generation of useful biomolecules. *Stenotrophomonas* sp. is known as an aerobic, non-fermentative, gram-negative bacterium capable of producing extracellular protease with broad specificities. The extracellular enzyme can degrade fibrinogen in the serum component completely, suggesting the potential of application in the process of blood clot modification (Windhorst *et al.* 2002). This premise has been demonstrated by the LK from earthworms *Lumbricus rubellus*, which is known for its high fibrinolytic activity (Nakajima *et al.* 1993). LK, as one of the commercial fibrinolytic agents produced by *L. rubellus*, was used to compare its fibrinolytic activity with that of the *Stenotrophomonas* sp. enzyme in our study. The result showed that our enzyme had similar fibrinolytic activity with the commercial LK at similar concentration applied, which strongly supports the potential application of fibrinolytic enzyme from this food origin *Stenotrophomonas* sp. The similar thrombus degrading effect of our *Stenotrophomonas* enzyme and that of LK had been also tested using experimental rats (Nailufar *et al.* 2016).

Many blood coagulation and fibrinolytic enzyme/factors are zymogens of serine proteinase, which hydrolyse specific peptide bonds (Devlin 1997). A variety of synthetic substrates are available for the analysis of activities of these proteins coagulation enzymes.

Thrombin is a serine protease involved in the coagulation cascade reactions, where it activates various protein factors and converts the soluble fibrinogen into insoluble strand of fibrin. As the final enzyme in the activation of coagulation system, thrombin is an important target for the development of new anticoagulant and/or antithrombotic drugs. This interest has led to finding of agents that have either a direct effect on the cleavage site of the thrombin molecule or can in some other way inhibit its ability to catalyse the conversion of fibrinogen to fibrin. Direct thrombin inhibitors are either derived from natural sources, such as hirudin or are chemically synthesized such as warfarin. Hirudin is a polypeptide originally obtained from leech. Hirudin acts by irreversible binding to the active site of thrombin. With their various nature and characteristics, thrombin inhibitors are used to

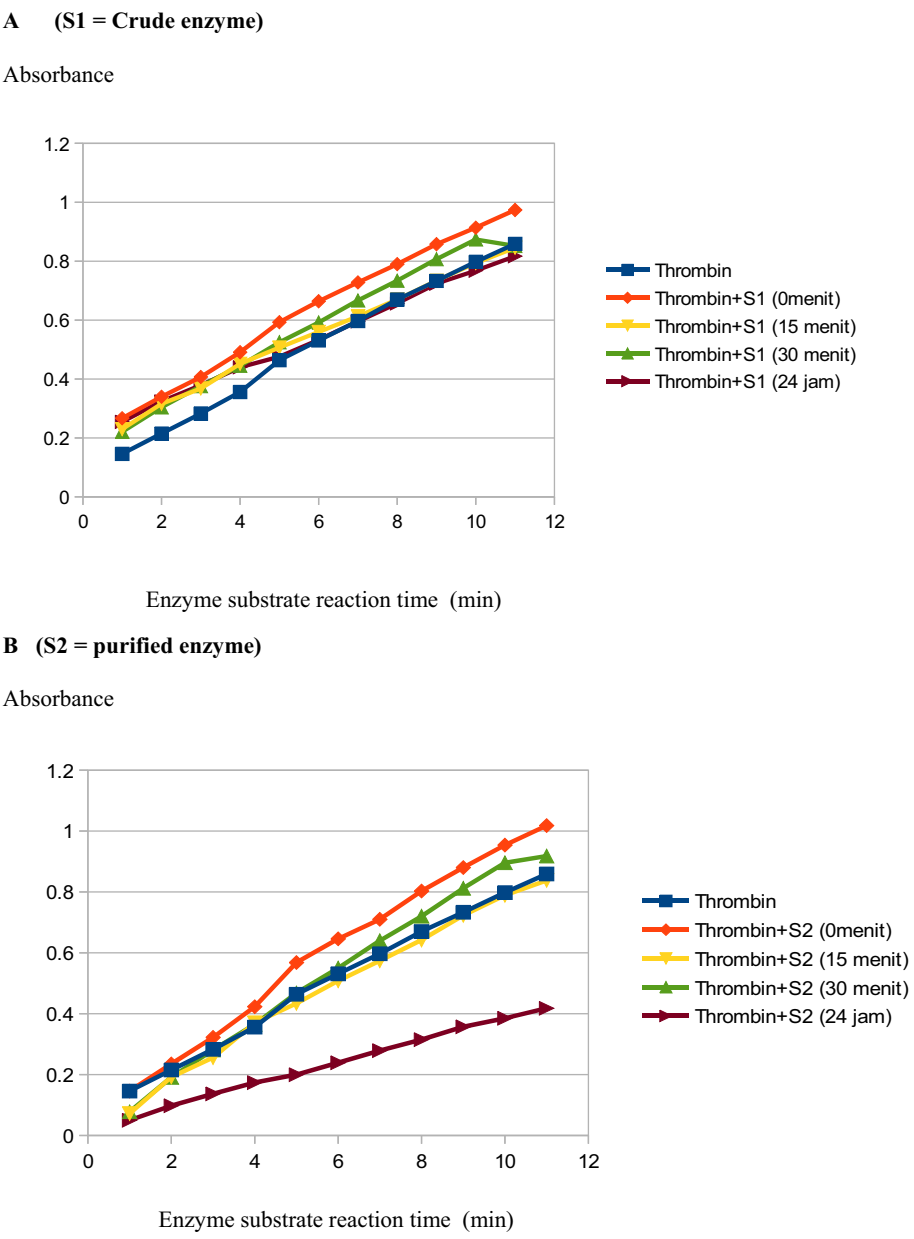


Figure 4. Reactivity of Chromozym TH to thrombin and thrombin treated with *Stenotrophomonas* sp. fibrinolytic enzyme. Determination of thrombin activity was conducted using Chromozym TH substrate. Enzyme was incubated with the crude (A) and purified enzymes (B) for 0 min, 15 min, 30 min and 24 h before incubation with the chromogenic substrate. The absorbance was measured for another 12 min as mentioned in the method.

prevent arterial and venous thrombosis. They can be used to prevent and treat deep vein thrombosis, or used as prophylaxis in atrial fibrillation to avoid thromboembolism (Fareed *et al.* 1999; Lee and Ansel 2011).

Thrombin activity may be detected using peptides that contain the VPR (Valin-Proline-Arginine) recognition sequence such as the fluorogenic substrates Boc-Val-Pro-Arg-AFC, Boc-Val-Pro-Arg-7-AMC or chromogenic substrate Boc-Val-Pro-Arg-pNA and Tos-Gly-Pro-Arg-4-pNA. Synthetic tripeptides (S-2160 and Chromozym TH) have been used as substrates for the measurement of thrombin activity. Using Chromozym TH (Tos-Gly-Pro-Arg-4-nitranilide acetate) which will release *p*-nitroanilide is qualified for assessing anti-thrombin activity (Devlin 1997). The purified enzyme of *Stenotrophomonas* showed inhibition of thrombin activity by 50% at 24 h incubation time. Discovery of new AT is still limited and thus interested to study. AT agents are also important for the treatment of patients with hereditary AT III deficiency in connection with surgical or obstetrical procedures or when the patients suffer from thromboembolism.

The purified *Stenotrophomonas* enzyme capable of hydrolysing fibrin and fibrinogen, as well as possessing anti-thrombin activity is a protein of approximately 31 kD. The molecular size is similar to that of BSN1 fibrinolytic protease from *B. subtilis* TKU007 (30 kD; Wang *et al.* 2011) and *B. subtilis* BK-17 (31 kD; Jeong *et al.* 2001). Fibrinolytic enzymes of different sizes from various microorganisms have been reported: 47 kD for streptokinase, 28 kD for nattokinase (Dubey *et al.* 2011), 28.2 kD for enzyme from *Bacillus* sp. strain CK11-4 (Kim *et al.* 1996) and 27.5 kD for enzyme from *B. subtilis* TP-6, isolated from Tempe (Kim *et al.* 2006).

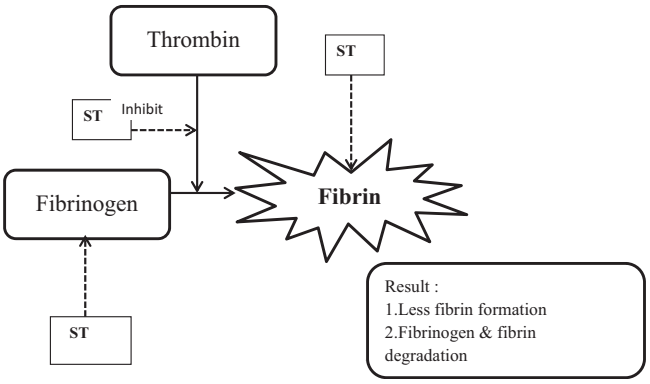


Figure 5. Fibrinolytic enzyme ST: possible mechanism of action in dissolving fibrin. Dotted arrow indicates hydrolysis mechanism, whereas flat arrow indicates the activation mechanism. Extracellular enzyme produced by *Stenotrophomonas* sp. (ST) isolated from Indonesian fermented food, *Oncom* can degrade fibrinogen and fibrin directly, whereas the purified enzyme of ST showed additional thrombin inhibiting activity.

In conclusion, we have shown fibrin and fibrinogen degrading activity of enzyme from *Stenotrophomonas* sp. isolated from Indonesian traditional fermented food *Oncom*. The purified enzyme was also found as an inhibitor of thrombin activity. This finding highlights the multiple actions of *Stenotrophomonas* enzyme. As an illustration, potential mode of action of *Stenotrophomonas* enzyme in fibrinolysis is presented in Figure 5.

Acknowledgements

This research was supported by Research Grant for Graduate Student 2014, Faculty of Biotechnology, Atma Jaya Catholic University. Proteolytic microorganisms from Oncom were collections of Diana Nur Afifah (PhD graduate of Bogor Agricultural University, under supervision of Maggy T Suhartono). Some of the work was conducted at DLBS (Dexa Laboratories for Biomolecular Science) Dexa Medica Jakarta. The authors would like to thank Ms Novita for her technical support.

Conflict of Interest Statement

The authors express no conflict of interest in regard to the content of this article.

References

Astrup T, Mullertz S. 1952. The fibrin plate method for estimating fibrinolytic activity. *Arch Biochem Biophys* 40:346–51.
Collen D, Lijnen HR. 2004. Tissue-type plasminogen activator: a histological perspective and personal account. *J Thromb Haemost* 2:541–6.
DeMers G, Meurer WJ, Shih R, Rosenbaum S, Vilke GM. 2012. Tissue plasminogen activator and stroke: review of the literature for the clinician. *J Emerg Med* 43: 1149–54.
Devlin TM. 1997. Serine proteases. In: Devlin TM (Ed.). *Textbook of Biochemistry With Clinical Correlations*, 4th edition. New York: Wiley, New York. pp. 97–108.
Dubey R, Kumar J, Agrawala D, Char T, Pusp P. 2011. Isolation, production, purification, assay and characterization of fibrinolytic enzymes (Nattokinase, Streptokinase and Urokinase) from bacterial sources. *African J Biotech* 10: 1408–20.
Duffy MU. 2002. Urokinase plasminogen activator and its inhibitor, PAI-1, as prognostic marker in breast cancer: from pilot to level 1 evidence studies. *Clin Chem* 48:1194–7.
Fareed J, Lewis BE, Callas DD, Hoppensteadt DA, Walenga JM, Bic RL. 1999. Antithrombin agents: the new class of anticoagulant and antithrombotic drugs. *Clin Appl Thromb Hemost* 5:S45–55.
Hua Y, Jiang B, Mine Y, Meng MW. 2008. Purification and characterization of a novel fibrinolytic enzyme from *Bacillus* sp. nov. SK006 isolated from an Asian traditional fermented shrimp paste. *J Agric Food Chem* 56:1451–7.
Jeong YK, Park JU, Baek H, Park SH, Kong IS, Kim DW, Joo WH. 2001. Purification and biochemical characterization of a fibrinolytic enzyme from *Bacillus subtilis* BK-17. *World J Microbiol Biotechnol* 17:89–92.
Kim HK, Kim GT, Kim DK, Choi WA, Park SH, Jeong YK, Kong IS. 1997. Purification and characterization of a novel fibrinolytic enzyme from *Bacillus* sp., KA38 originated from fermented fish. *J Ferment Bioeng* 84:307–12.
Kim SB, Lee DW, Cheigh CL, Choe EA, Lee SJ, Hong YH, Choi HJ, Pyun YR. 2006. Purification and characterization of a fibrinolytic subtilisin-like protease of *Bacillus subtilis* TP-6 from an Indonesian fermented soybean, Tempeh. *J Ind Microbiol Biotech* 33:436–44.
Kim SH, Choi NS. 2000. Purification and characterization of subtilisin DJ-4 secreted by *Bacillus* sp. strain DJ-4 screened from Doen-Jang. *Biosci Biotech Biochem* 64: 1722–5.
Kim W, Choi K, Kim Y. 1996. Purification and characterization of a fibrinolytic enzyme produced from *Bacillus* sp. strain CK 11-4 screened from Chungkook-Jang. *Appl Environ Microbiol* 62:2482–8.
Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 22:680–5.
Lee CJ, Ansell JE. 2011. Direct thrombin inhibitors. *British J Clin Pharmacol* 72: 581–92.
Li W, Johnson DJ, Esmon CT, Huntington JA. 2004. Structure of the antithrombin-thrombin-heparin ternary complex reveals the antithrombotic mechanism of heparin. *Nat Struct Mol Biol* 11:857–62.
Lioudaki EL, Ganotakis ES. 2010. Associations of thrombotic-hemostatic factors with cardiovascular disease. *J Clin Chem* 3:25–37.
Lowry OH, Rasebrough NJ, Farr A, Randal RJ. 1951. Protein measurement with folin phenol reagent. *J Biol Chem* 193:256–75.
Miyaji T, Otta Y, Shibata T, Mitsui K, Nakagawa T, Watanabe T, Niimura Y, Tomizuka N. 2005. Purification and characterization of extracellular alkaline serine protease from *Stenotrophomonas maltophilia* strain S-1. *Lett Appl Microbiol* 41:253–7.
Nailufar F, Tjandrawinata RR, Suhartono MT. 2016. Thrombus degradation by fibrinolytic enzyme of *Stenotrophomonas* sp. originated from Indonesian soybean-based fermented food on Wistar rats. *Adv Pharmacol Sci*. https://doi.org/10.1155/2016/4206908.
Nakajima N, Mihara H, Sumi H. 1993. Characterization of a fibrinolytic enzymes in earthworm, *Lumbricus rubellus*. *Biosci Biotechnol Biochem* 57:1726–30.
Peng H, Huang Q, Zhang RH, Zhang YZ. 2003. Purification and characterization of a fibrinolytic enzyme produced by *Bacillus amyloliquefaciens* DC-4 screened from *douchi*, a traditional Chinese soybean food. *Comp Biochem Physiol Part B Biochem Mol Biol* 134:45–52.
Sugimoto S, Fujii T, Morimiya T, Johdo O, Nakamura T. 2007. The fibrinolytic activity of a novel protease derived from a Tempeh producing fungus *Fusarium* sp. BLB. *Biosci Biotechnol Biochem* 71:2184–9.
Voet D, Voet JG. 1990. Biochemistry, 2nd edition. New York: Wiley. pp. 1087–95.
Wang C, Ji B, Li B, Ji H. 2006. Enzymatic properties and identification of a fibrinolytic serine protease purified from *Bacillus subtilis* DC33, isolated from Chinese traditional *Douchi*. *J Ind Microbiol Biotechnol* 33:750–8.
Wang C, Ming D, Dongmei Z, Fandong K, Guoren Z, Yibing F. 2009. Purification and characterization of nattokinase from *Bacillus subtilis* Natto B-12. *J Agric Food Chem* 57:9722–9.
Wang SL, Wu YY, Liang TW. 2011. Purification and biochemical characterization of a nattokinase by conversion of shrimp shell with *Bacillus subtilis* TKU007. *New Biotechnol* 28:196–200.
Windhorst S, Frank E, Georgieva DN, Genov N, Buck F, Borowski P, Weber W. 2002. The major extracellular protease of the nosocomial pathogen *Stenotrophomonas maltophilia*: characterization of the protein and molecular cloning of the gene. *J Biol Chem* 277:11042–9.